P-glycoprotein (P-gp) is upregulated in peripheral T-cell subsets from solid organ transplant recipients

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Immunosuppressive agents such as cyclosporine, tacrolimus, sirolimus, and corticosteroids are substrates for the transmembrane multidrug resistance pump P-glycoprotein (P-gp). Experience in oncology has suggested that chronic exposure to P-gp substrates induces upregulation of P-gp activity, which could result in resistance to immunosuppressive drugs. The authors investigated P-gp function in CD4+ and CD8+ T cells from the peripheral blood of solid organ transplant recipients (SOTX). Subjects included 14 stable SOTX (10 liver, 4 lung) and 16 healthy controls. Four-color flow cytometry was used to simultaneously measure intracellular concentration of the fluorescent P-gp substrate Rhodamine 123 (Rh123) and surface expression of CD45RO (nominal memory/effector), CD45RA (naive), and either CD4 or CD8. P-glycoprotein function was measured by a dye efflux assay in which activity was inferred from a decrease in Rh123 fluorescence. CD4+ and CD8+ T cells from patients and control subjects eliminated Rh123, and this activity was inhibited by verapamil, a known P-gp substrate. CD8+ T cells had greater P-gp activity than CD4+ cells, and naive and transitional T cells displayed greater activity than memory T cells. Activity was bimodal in CD8+ CD45RO+ T cells, with a subset of these cells expressing the greatest P-gp activity. Patient CD8+ naive and transitional T cells had upregulated P-gp activity compared to control subjects. We conclude that (1) P-gp activity is significantly upregulated in specific T-cell subsets (CD8+/CD45RA+) in the peripheral blood of SOTX, and (2) the bimodal nature of P-gp response in CD8+ T cells complicates analysis of the effect of chronic administration of P-gp substrates to SOTX.

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Despite these observations, the role of P-gp in immune function remains unclear. P-glycoprotein has been associated with organ transplant rejection in a number of clinical settings. Upregulation of P-gp expression in the intestine could result in lowered absorption of immunosuppressive agents that are P-gp substrates. Such an effect would be manifest by lower plasma concentrations of these drugs. In addition, upregulation of P-gp in the target cell population (T cells) could result in decreasing intracellular drug exposure, rendering these cells resistant to therapy in the face of adequate plasma drug concentrations. Both mechanisms could reduce the therapeutic efficacy of a wide range of drugs used to prevent or treat rejection.

Therefore, the major objective of our study was to compare P-gp activity in peripheral blood T cell subsets from healthy control subjects and solid organ transplant patients to determine whether the drug treatment of solid organ transplant patients is associated with a detectable increase in P-gp activity.

PATIENTS AND METHODS

Subjects

In this cross-sectional study, 14 solid organ transplant recipients (10 liver, 4 lung) were studied at a mean of 75 days posttransplant (range: 7–207 days). Two liver transplant recipients were studied on two occasions each, and 1 lung transplant recipient was studied on three occasions. All patients received tacrolimus and corticosteroids as primary immunosuppression. Healthy control subjects (16 individuals, 17 observations) were assayed concurrently with all patient samples. Then, 10 ml of heparinized (10 units/ml, from beef lung, Upjohn) blood were obtained from each subject studied. Informed consent was obtained from all subjects according to a protocol approved by the University of Pittsburgh's institutional review board.

Measurement of P-gp Function by Flow Cytometry

Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation over Ficoll-Hypaque gradients (Sigma Diagnostics, Inc., St. Louis, MO) according to the manufacturer’s instructions. One million PBMC were suspended in 1 ml of complete medium (CM) consisting of RPMI1640 supplemented with L-glutamine (200 µM), penicillin (100 U/ml), gentamicin (50 µg/ml), and HEPEs buffer (10 mM). Cells were loaded with Rhodamine 123 (Rh123, Sigma Chemicals, 3 µl of 150 ng/ml) to yield 0.45 ng/ml final concentration and incubated for 45 minutes at 37°C in the presence or absence of verapamil (50 µM). Verapamil was used as a P-gp specific competitive inhibitor to estimate the amount of Rh123 retention in the absence of P-gp activity (Figure 1). At the end of the “loading” incubation, cells were washed twice with 10 ml of ice-cold phosphate buffered saline without Ca++ and Mg++. Washed cells were resuspended in 1 ml of CM plus 10% human AB serum. Rh123 was then allowed to efflux for 45 minutes at 37°C. For cells loaded with Rh123 in the presence of verapamil, 50 µM verapamil was maintained during the wash and the efflux period. Immediately after the efflux period, cells were stained on ice for phenotypic expression of surface CD4 (ECD conjugated, Beckman-Coulter 6604727) or CD8 (ECD conjugated Beckman-Coulter 6604728), plus CD45RA (PE conjugated, Beckman-Coulter 6603181) and CD45RO (PE Cy5, Caltag Laboratories, Burlingame, CA, MHC45RO06). After a 20-minute incubation on ice, cells were washed and acquired immediately on a four-color Coulter XL flow cytometer. This technique allowed simultaneous measurement of the intracellular concentration of the fluorescent P-gp substrate Rh123, as well as surface expression of CD45RO (a nominal memory marker) and CD45RA (naive marker) on either CD4+ or CD8+ lymphocytes. Lymphocytes were identified as low log side scatter and low forward scatter events. P-glycoprotein activity was determined as the difference in Rh123 mean log fluorescence intensity in the presence or absence of verapamil.

Statistical Analysis

Paired comparisons of ranked values were made using the Wilcoxon sign rank test (two-tailed) since the differences in fluorescence intensity in the presence and absence of the P-gp specific inhibitor verapamil were not normally distributed. The Mann-Whitney U test was used to compare the difference in Rh123 mean fluorescence intensity between patient and control groups. An unpaired two-tailed t-test was used to compare CD45 isoform expression in patient and control groups; p-values ≤ 0.05 were considered statistically significant.

RESULTS

Use of Multiparameter Flow Cytometry to Measure P-gp Function in T Cell Subsets

We have adapted published methods in which the fluorescent P-gp substrate Rh123 is used to probe P-gp ac-
activity in resting lymphocytes. The use of multiparameter flow cytometry allows P-gp function to be measured concurrently with three other fluorescence parameters defining T-cell subsets. Figure 1 shows the simultaneous assessment of Rh123 efflux in CD4+ and CD8+ T cells. An additional parameter (CD45RA) was also measured but is not shown in this figure. CD4+ cells effluxed Rh123 as a single asymmetric peak. CD8+ cells expelled more Rh123 than CD4+ cells and showed two populations with distinct pumping activity (p < 0.05). Both T-cell subsets were comparably bright in the presence of the specific P-gp inhibitor verapamil, indicating similar inherent capacity for Rh123 uptake and confirming that P-gp was responsible for Rh123 efflux.

Figure 2 shows a similar experiment in which Rh123 efflux was measured in T cells subsetted on the basis of CD45 isoform expression (CD45RA vs. CD45RO) and either CD4 (left panel) or CD8 (right panel). Among CD4+ T cells, CD45RA+/RO− (naive) have greater P-gp activity than their CD45RA-/RO+ (memory) counterparts (p < 0.05). In CD8+ T cells, the situation is more complex, as Rh123 staining was essentially unimodal in CD45RA+/RO− cells but was bimodal among CD45RA-/RO+. As in the previous experiment (Figure 1), inclusion of verapamil resulted in uniform high retention of Rh123, indicating that P-gp was responsible for dye efflux. These results indicate inherent differences in P-gp activity relating to T cell subset and differentiation state. The average P-gp activity was higher in naive (CD45RA+/CD45RO−) than in memory (CD45RA−/RO+) T cells, but the cells with the greatest activity were found in the leftmost population of the bimodally distributed CD45RO+ CD8+ T cells.

P-gp Activity in Lymphocytes from Transplant Recipients and Controls

Table I and Figure 3 summarize P-gp activity by T-cell subset in healthy control subjects and transplant recipients. In addition to the CD45RA+/RO− and CD45RA-/RO+ subsets shown in Figure 2, we also identified T-cells expressing both isoforms (transitional). Qualitatively, the T cell subsets from control
subjects and patients behaved in a similar fashion in that CD8 cells displayed more activity than CD4 cells. Among CD8+ T cells, CD45RA+/RO– and transitional cells were more active Rh123 pumpers than CD45RA–/RO+.

When P-gp activity in T cells from transplant patients is compared to that of healthy control subjects (Figure 3), results are indistinguishable for CD4+ T cells (all subsets). In contrast, CD8+ T cells from patients displayed increased P-gp activity. In CD45RA+/RO– and transitional subsets, the difference in P-gp activity attained statistical significance (p = 0.035 and 0.009, respectively).

CD4+ and CD8+ T cells were expressed as a percentage of lymphocytes (as defined by forward and log side scatter). Transplant patients had a significantly smaller proportion of CD4+ T cells (18.6% to 32.2%, lower/upper 95% confidence intervals), compared to control subjects (35.2% to 43.3%, p = 0.001, Student’s t-test). Figure 4 compares the distribution of CD45 isoforms between patients and controls. In CD4+ T cells, patients had a marked skewness toward increased memory/effector T cells. Therefore, they had a significantly smaller proportion of CD45RA+/RO– cells and a correspondingly greater proportion of transitional (CD45RA+/CD45RO+) plus CD45RA–/CD45RO+ cells. A similar situation was not observed for CD8+ T cells.

DISCUSSION

This study demonstrates that T cells from patients receiving chronic immunosuppressive therapy upregulate multidrug resistance pump activity in some T-cell subsets compared to T cells from healthy control subjects. The most pronounced dye efflux was seen in CD8+ effector T cells. The ability of such T cells to upregulate a pump that excludes immunosuppressive agents has implications for therapy of graft rejection. The literature provides conflicting data bearing on the hypothesis that the expression of the MDR1 gene prod-
uct P-gp represents a mechanism of therapy-resistant rejection in solid organ transplant patients. Gotzl et al\textsuperscript{15} collected the peripheral blood cells of 32 patients following renal transplantation and measured P-gp expression by MDR1 RNA analysis. Of the 32 patients tested, 9 patients (28\%) expressed high levels of MDR1 RNA in their lymphocytes. In 6 patients who received their kidney transplant more than 40 months prior, no MDR1 RNA could be detected, suggesting that the lack of MDR expression may coincide with prolonged graft survival. In contrast, Melk et al\textsuperscript{16} found no difference in P-gp expression in PBMC from kidney transplant patients with stable grafts versus those with chronic rejection. Similarly, Delaney et al\textsuperscript{17} classified renal transplant patients into cyclosporine/tacrolimus sensitive or resistant based on the in vitro sensitivity of their peripheral T cells to these drugs. They found no difference in P-gp activity of T cells in these groups and concluded that increased P-gp activity could not explain drug resistance. In contrast to these reports, P-gp expression in PBMC from heart transplant recipients has been associated with acute rejection.\textsuperscript{18} P-gp was detected by immunocytochemistry in PBMC from 32 of 49 patients in this study. A significant increase was seen in the incidence of acute rejection in patients with increasing numbers of P-gp+ PBMC over the 2-year interval of observation.

In a study from the University of Pittsburgh, P-gp expression was measured using P494 murine monoclonal antibody\textsuperscript{19} in mononuclear inflammatory cells from transbronchial or open lung biopsy specimens of lung allograft recipients. Patients were grouped into those who experienced steroid-sensitive or steroid-resistant episodes of acute rejection or who experienced bronchiolitis obliterans. The expression of P-gp was significantly higher in steroid-resistant patients and in patients with bronchiolitis obliterans than in steroid-sensitive patients studied during episodes of acute rejection. Based on these observations, P-gp expression may be related to the failure of steroid-induced immunosuppression and may predict a poor prognosis.

The method used to detect P-gp also has an impact on the present results. We chose the Rh123 efflux assay for the present study because it is able to detect the activity of drug efflux proteins at the low levels of drug resistance that are likely to be attained in vivo.\textsuperscript{20} Although monoclonal antibodies such as UIC2 (Beckman-Coulter) and MRK16 (Kamiya) are commercially available, the quantitative analysis of P-gp in freshly isolated T cells is difficult. A considerable overlap in fluorescence intensity between experimental and isotype control samples has been observed by all investigators, leading them to conclude either that de-

<table>
<thead>
<tr>
<th>Group</th>
<th>T Cell Subset</th>
<th>CD45 Subset</th>
<th>VERA – No VERA (median difference of mean fluorescence intensity)</th>
<th>p-Value (VERA vs. no VERA)</th>
<th>P-gp Activity\textsuperscript{a}</th>
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<tr>
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<td>0.001</td>
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<td>CD8</td>
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<td>27.3</td>
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<tr>
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<td>0.001</td>
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<td>29.9</td>
<td>0.000</td>
<td>++, +++\textsuperscript{b}</td>
</tr>
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\textsuperscript{a} P-gp activity was defined in relation to the pooled differences (VERA vs. no-VERA) in all T cell subsets. All subsets displayed P-gp activity. Each subset was assigned a relative activity: + (low) indicates the median value lies within the second quartile, ++ (moderate) within the third quartile, and +++ (high) within the fourth quartile.

\textsuperscript{b} P-gp activity was bimodal in CD8+ CD45RA–/RO+ T cells.
tection is not possible or that it is necessary to use controversial statistical methods to detect specific antibody binding. Despite the fact that the quantity of P-gp expressed on resting T cells has been estimated to be several orders of magnitude lower than in drug-resistant cell lines or transfected cells, P-gp activity is easily demonstrated by dye exclusion (calcein acetoxymethyl ester) or dye efflux (Rh123) assays. We chose the latter because the excitation and emissions spectra of Rh123 is similar to that of fluorescein, permitting it to be used in conjunction with three different dyes that are commercially available conjugated to murine monoclonal antibodies.

The fluorescent dye Rh123 is itself a P-gp substrate. Therefore, Rh123 loading and efflux can be directly measured by incubating cells in the presence of Rh123, with washing and measuring fluorescence after 15 to 120 minutes in culture. The major disadvantage of Rh123 as a measure of P-gp activity is that, once inside the cell, a proportion of Rh123 accumulates in mitochondria, where it is unavailable for export by P-gp. A corollary is that maximal Rh123 uptake is related to the number and activity of mitochondria and therefore cellular metabolic activity. Fortunately, metabolic activity is low and stable in the short-term culture of resting T cells. Therefore, the difference in fluorescence after incubation in the absence and presence of a competitive inhibitor (verapamil) provides a reliable indicator of multidrug resistance pump activity.

Several lines of evidence indicate that, in resting T cells, the Rh123 dye efflux assay is specific for P-gp. Dye efflux can be abolished by the monoclonal anti-
body UIC2,24,25 or by competitive inhibition with high concentrations of P-gp substrate drugs such as verapamil.26 In addition to P-gp, mRNA for other transporters such as cMOAT/MRP2, LRP,27 and MDR128 have been detected in normal peripheral blood mononuclear cells. Of these, cMOAT is not sensitive to inhibition by verapamil,29 ruling out its activity in the present study. The effect of verapamil on LRP is unclear. Lamy et al30 attributed verapamil-insensitive Rh123 pumping in two cases of large granulocytic leukemia to LRP activity. We have shown that genistein, an MRP inhibitor that is not a P-gp substrate,31 does not inhibit Rh123 dye efflux in normal human T cells.32 Although our data do not permit us to formally rule out the participation of other transporters, we have inferred P-gp function based on the presumed specificity of the Rh123/verapamil assay in normal resting T cells.

Both CD4+ and CD8+ T cells from control subjects eliminated Rh123 as evidenced by low fluorescence. In agreement with published observations,11,28,33,34 CD8+ T cells demonstrated more P-gp activity than CD4+ T cells. We have extended these findings by demonstrating that CD8+ cells pump in a bimodal fashion with high-pumping and low-pumping subpopulations, whereas P-gp activity is more homogeneous in CD4+ T cells.

Naive, memory, and effector T cells differ with respect to their biological activity, in vivo half-life, and susceptibility to immunosuppressive agents. In the present study, we sought to determine whether there are commensurate differences in P-gp function. We used four-color flow cytometry on T-cell subsets (CD4 and CD8) further divided on the basis of CD45 isoform expression to determine the intracellular concentra-
tation of the fluorescent P-gp substrate Rh123. CD4 and CD8 T cells were separated into CD45RA−/RO+ (nominal memory/effecter), CD45RA+/RO− (naive), and a transitional population expressing both isoforms. P-gp function was inferred from an increase in Rh123 concentration in the presence of verapamil. Using two-color flow cytometry, Chaudhary et al11 also examined P-gp function in lymphocytes subsetted on the basis of either CD45RO or CD45RA and found activity to be highly heterogeneous in these subsets. We were able to explain this heterogeneity through the use of four-color flow cytometry and resolution of six distinct T-cell subsets (CD4 and CD8, each with three subsets of CD45 isoform expression). In addition to resolution of populations that have been associated with naive and memory/effecter subsets, we also resolved a transitional population that expressed both CD45RA and CD45RO. This phenotype is seen on activated T cells in vivo35 and in vitro (VSD, unpublished results). Since this population expresses both CD45 isoforms, it will always score as positive if a single parameter (CD45RA or CD45RO) is used to define CD45 subsets. As shown in Table I, CD8+ CD45RA+ T cells had the greatest P-gp activity, followed by transitional CD8+ and then by CD45RO+ cells. However, the P-gp activity was bimodal among CD8+ CD45RO+ cells, including a minor subpopulation with the highest P-gp activity. Studies now in progress evaluating graft-infiltrating T cells from lung allografts suggest that this subpopulation includes activated effector cells expressing HLA-DR, CD25, and CD71, which are all markers of in vivo activation. In the present study, we observed analogous differences in P-gp activity among CD4 subsets (Table I).

P-gp activity in the T-cell subsets of healthy control subjects serves as a point of reference to evaluate P-gp response in transplant recipients (Figure 3). In the patient group, Rh123 elimination was highly sensitive to verapamil blockade in all T-cell subsets, indicating high P-gp activity. Although the median responses in cells from patients were uniformly higher than those observed in controls, differences between patients and controls were statistically significant only in CD8+/CD45RA+ and CD8+ CD45RA+/CD45RO T-cell subsets. This increase in P-gp activity was observed despite the fact that all patients received both tacrolimus and corticosteroids. Since these agents are P-gp substrates, they could potentially act as competitive inhibitors of Rh123 efflux. However, blood concentrations of tacrolimus ranged from 5.7 to 16.6 ng/ml (6.9 to 20.2 nm), and prednisone doses were 0 to 50 mg/day. Thus, it is unlikely that in vivo exposure to these agents at these concentrations resulted in competitive inhibition in vitro.

Patients also differed in the proportion of CD4+ T cells and in the proportion of naive T cells, which were both lower in patients (Figure 4). The corresponding increase in CD8+ memory/effecter cells, coupled with high inherent P-gp activity, may be a direct consequence of the selective pressure imposed by immunosuppressive therapy. Alternatively, the emergence of memory effector CD8 cells with high P-gp activity may reflect the generation of apoptosis-resistant cells, as has been demonstrated in cell lines transfected with P-gp or selected for drug resistance.36 This mechanism may render organ transplant recipients resistant to long-term immunosuppression. Finally, P-gp upregulation in the CD8+ memory/effecter subset may be the consequence of T-cell activation in response to alloantigen and therefore not directly related to drug exposure.

In conclusion, we have demonstrated that P-gp activity is upregulated in specific T-cell subsets (CD8+/CD45RA+) in peripheral blood in SOTX patients. This study has also demonstrated that P-gp activity is higher in CD8+ than in CD4+ T cells from solid organ transplant patients. The bimodal nature of the P-gp response in CD8+ T cells further complicates the analysis of the effect of the chronic administration of P-gp substrates to solid organ transplant patients. Future studies must take these factors into account when assessing the relationship between P-gp expression in T cells and the outcome of immunosuppressive therapy in organ transplant patients.

REFERENCES

P-gp UPREGULATION IN PERIPHERAL T-CELL SUBSETS


